

plant transformed (Example 5) with the expression vector pPV.8-GBSS-HCV33-myc (of Example 1). The positive control was at a concentration of 20µg/ml. Total protein concentration in the plant extract was 1-2mg/ml.

Figure 4 shows the results of immuno-histology studies, as reported in Example 5, on a tobacco plant transformed with the expression vector pPV.8-GBSS-HCV33-myc (of Example 1), confirming that HCV33 fragment is localised in the chloroplasts.

Figure 5 shows the amount of active antibody present in fruit columella extracts of a tomato plant transformed (Example 5) with the plant expression vector pPV.8-GBSS-HCV33-myc (of Example 1), expressed as % of total soluble protein, as determined via direct binding ELISA.

Figure 6 shows the nucleotide sequence (SEQ. ID. No. 4) and corresponding protein sequence (SEQ. ID. No. 5) of a fragment encoding the heavy chain variable domain of an anti-potato SBEII antibody (denoted Clon1) with attached peptide linker groups denoted His6 (His-His-His-His-His-His) and myc (as described in Figure 1). (Potato SBEII is a starch branching enzyme also denoted SBE A; Jobling *et. al.*, Plant Journal, 18, 163-171 1999).

Figure 7 shows the amount of active antibody present in whole leaf extract of a tobacco plant transformed (Example 5) with the plant expression vector pSJ35-CERV-GBSS-Clon1-His6-myc (of Example 1), expressed as % of total soluble protein, as determined via direct binding ELISA.

Figure 8 shows the results of immuno-histology studies as reported in Example 6 on a tobacco plant transformed with the expression vector pSJ.34-2x35S-GBSS-scFv3299-hydrophilIII (of Example 1). It is clear that plastid formation/function is disrupted.

Figure 9 shows the amount of active antibody present in leaf extract of tobacco plants transformed (Example 7) with the plant expression vector pPV.8-PR1a-HCV33-myc (of Example 1), expressed as % of total soluble protein, as determined via direct binding ELISA.

Figure 10 shows a specificity ELISA assay (A) and SDS-PAGE/western blot analysis (B) of whole cell extracts of tobacco plants transformed (Example 8) with two plant expression vectors, namely pPV.8-PR1a-HCV33-myc-SEKDEL and pPV.8-GBSS-HCV33-myc-SEKDEL (of Example 1). Total protein concentration in the plant extract was 1-2mg/ml.

Figure 11 shows the results of immuno-histology studies as reported in Example 8 on a tobacco plant transformed with the expression vector pPV.8-PR1a-HCV33-myc-SEKDEL (of Example 1) confirming that the HCV33 material, directed to the secretory pathway using the PR1a leader sequence, and retained in the endoplasmic reticulum by the C-terminal tag SEKDEL.

Figure 12 shows the nucleotide sequence (SEQ. ID. No. 6) and corresponding protein sequence (SEQ. ID. No. 7) of a fragment encoding the heavy chain variable domain of an anti-potato SBEII antibody (denoted Clone46) with attached peptide linker groups denoted His6 and myc (as described in Figure 1).

Figure 13 shows that the SBE (starch branching enzyme) activity of whole leaf extracts of 30 tobacco plants independently transformed (Example 9) with the plant expression vector pSJ.35-CERV-GBSS-Clone46-His6-myc (of Example 1) is significantly less than that of plants transformed with pSJ.35-CERV-GBSS-HCV33-myc (of Example 1).

Figure 14 shows the nucleotide sequence (SEQ. ID. No. 8) and corresponding protein sequence (SEQ. ID. No. 9) of a fragment encoding the heavy chain variable domain of an anti-GUS antibody (denoted Clone18) with attached peptide linker groups denoted His6 and myc (as described in Figure 1). (GUS is  $\beta$ -glucuronidase, available from Sigma.)

Figure 15 shows the nucleotide sequence (SEQ. ID. No. 10) and corresponding protein sequence (SEQ. ID. No. 11) of a fragment encoding the heavy chain variable domain of an anti-GUS antibody (denoted Clone18) with an endoplasmic retention signal (SEKDEL) attached peptide linker groups denoted His6 and myc (as described in Figure 1).

Figure 16 shows that the GUS ( $\beta$ -glucuronidase) activity of whole leaf extracts of 30 pCJ.102 (of Example 1) transformed tobacco plants independently re-transformed (Example 10) with the plant expression vector pSJ.35-CERV-Clone18-His6-myc-SEKDEL (of Example 1) is significantly less than that of control pCJ.102 plants.

5 Figure 17 shows (A) X-glu staining of pollen from a tobacco plant transformed (Example 11) with pSJ.35-Lat52-HCV33-myc-SEKDEL (of Example 1), and (B) X-glu staining of pollen from a tobacco plant transformed (Example 11) with pSJ.35-Lat52-Clone18-His6-myc-SEKDEL (of Example 1).

10 Figure 18 shows the nucleotide sequence (SEQ. ID. No. 54) and corresponding protein sequence (SEQ. ID. No. 55) of a fragment encoding the heavy chain variable domain of an anti-RR6 antibody (denoted HCV33) linked to the non-classical heavy chain constant regions (denoted hinge-CH2-CH3).

15 Figure 19 shows the nucleotide sequence (SEQ. ID. No. 56) and corresponding protein sequence (SEQ. ID. No. 57) of a fragment encoding the heavy chain variable domain of an anti-RR6 antibody (denoted HCV33) linked to the non-classical heavy chain constant regions (denoted Hinge-CH2-CH3) with an endoplasmic retention signal (SEKDEL).

20 Figure 20 shows the amount of active antibody present in whole leaf extract of a tobacco plant transformed (Example 12) with the plant expression vector pPV.8-HCV33-IgG2b (of Example 12), expressed as % of total soluble protein as determined via direct binding ELISA.

25 Figure 21 shows the amount of active antibody present in whole leaf, stem and root extract of three tobacco plants (from Figure 20) analysed 6 weeks post germination, expressed as % of total soluble protein as determined via direct binding ELISA (of Example 12).

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Figure 22 shows the amount of active antibody present in whole leaf extract of a tobacco plant transformed (Example 12) with the plant expression vector pPV.8-GBSS-HCV33-IgG2b (of Example 12), expressed as % of total soluble protein as determined via direct binding ELISA.

Figure 23 shows the amount of active antibody present in whole leaf, stem and root extract of three tobacco plants (from Figure 22) analysed 6 weeks post germination, expressed as % of total soluble protein as determined via direct binding ELISA (of Example 12).

Figure 24 shows the amount of active antibody present in whole leaf extract of a tobacco plant transformed (Example 12) with the plant expression vector pPV.8-PR1a-HCV33-IgG2b (of Example 12), expressed as % of total soluble protein as determined via direct binding ELISA.

Figure 25 shows the amount of active antibody present in whole leaf, stem and root extract of three tobacco plants (from Figure 24) analysed 6 weeks post germination, expressed as % of total soluble protein as determined via direct binding ELISA (of Example 12).

Figure 26 shows the amount of active antibody present in whole leaf extract of a tobacco plant transformed (Example 12) with the plant expression vector pPV.8-PR1a-HCV33-IgG2b-SEKDEL (of Example 12), expressed as % of total soluble protein as determined via direct binding ELISA.

Figure 27 shows the amount of active antibody present in whole leaf, stem and root extract of three tobacco

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plastid, cytoplasm, apoplast and endoplasmic reticulum. Selfing of transformed, antibody-expressing plants produced a new generation of plants, many of which displayed an ability to accumulate higher levels of antibody than the parent plants. The amounts of antibody were found to be markedly greater than those typically achieved when conventional antibodies are expressed in plants. The accumulation of high levels of functional antibody in the cytoplasm was a particularly unexpected result, since conventional antibodies have generally been found to be produced only in very small amounts in this location, probably owing to the problem of forming the disulphide bonds needed for structural stability in a reducing environment.

The method of the invention was similarly found to be capable of producing VHH fragments in significant amounts in these different compartments. This was the case for several different VHH fragments, in contrast to the situation with fragments based on conventional antibodies, such as scFv, where the results have been found to be very unpredictable. Importantly, it was found that the accumulation of VHH fragments in any compartment of the cell resulted in no discernible deleterious effect on the plant; again this contrasts with results obtained with scFv fragments, where expression levels were found to be unstable and accumulation of the fragment in the plastids was found to affect adversely the structure and function of these organelles.

By means of the invention, antibodies and active fragments thereof, having specificity for a target antigen may conveniently be prepared by a method which is readily amenable to industrial-scale production. Further, the ability to target the antibodies to any chosen cell compartment makes it possible to use the method of the invention to modulate the

activity of specific enzymes irrespective of where in the cell the enzyme is found.

In order to modify the function of an intracellular protein, the antibody or fragment or derivative thereof must be present and active in those compartments of the host plant cell where the target protein is active. In cases where this is the cytoplasm, the reducing environment inhibits the formation of disulphide links in the antibody and so generally antibodies would not be expected to be active in the cytoplasm. Expression of classical antibodies, or fragments thereof, in the cytoplasm does not generally give rise to functional actively, for example. Similarly, expression of scFv fragments in plastids generally leads to unstable deformed plastids. The targeted expression of active antibodies or fragments or derivatives thereof in compartments of the host plant cell, especially in the cytoplasm, therefore represents a particular advantage of the method of the invention.

The present inventors have found that even when the levels of expression of heavy chain immunoglobulin or fragment or derivative thereof in the plant cell are low or undetectable by conventional protein chemical methods such as in the case of expression in the cytoplasm, a measurable effect on enzyme activity may still be achieved. The ability to modulate plant enzyme activity without accumulating antibodies in large amounts may be beneficial in some circumstances, for example, in minimising potential deleterious effects on the growth and health of the plant.

The heavy chain immunoglobulins (or fragments or derivatives thereof) to be expressed in accordance to the invention are, or are derived from, immunoglobulins that are naturally devoid of light chains, their antigen binding sites being contained entirely within the heavy chain variable domains. Especially



preferred are immunoglobulins or fragments or derivatives thereof which are obtainable from Camelids as described, for example, in WO 94/4678 above. Heavy chain variable domains of immunoglobulins naturally devoid of light chains are particularly preferred for use in the present invention.

Preferred immunoglobulins for use in the invention are obtainable from *Camelids* especially from *Lamas* (for example *Lama glama*, *Lama vicugna* or *Lama Paccos*) or from *Camelus* (for example *Camelus dromedarius* or *Camelus bactrianus*).

Conveniently, a functionally equivalent protein shows at least 50% similarity to the respective amino acid sequence, preferably at least 70% similarity, more preferably at least 80%, most preferably 90-100% similarity to the respective amino acid sequence as determined by techniques well known in the art.

The invention also provides for the expression of complex derivative molecules comprising an immunoglobulin or fragment thereof as described above, associated or otherwise connected to one or more other molecules, for example, an enzyme or a further heavy chain variable domain.

The invention provides specifically for the targeting of expressed antibodies, or fragment or derivatives thereof, to specific cellular compartments. Where the desired compartment is the cytoplasm, this is the default location when no specific targeting sequence is appended to the antibody. For targeting to other compartments, sequences encoding appropriate targeting signals are fused to the sequence encoding the antibody.

Where it is desired to target an antibody to the chloroplast, a sequence encoding an appropriate transit peptide is provided, such that said peptide will be fused at the N-terminus of the expressed antibody and subsequently removed upon passage into the organelle. Any chloroplast-specific transit peptide may be used together, optionally, with a few (1-3) residues from the N-terminus of the mature protein to which said peptide is fused in nature. A number of such sequences, proven to be capable of directing successful targeting of heterologous proteins, are well known to those skilled in the art. Particularly preferred is the GBSS transit peptide sequence from potato (van der Leij et al (1991) Mol. Gen. Genet., 228, 240-248).

Where it is desired to direct secretion of an antibody into the apoplastic space, a sequence encoding an appropriate signal peptide is provided, such that said peptide will be fused at the N-terminus of the expressed antibody and subsequently removed upon passage into the endoplasmic reticulum, en route to secretion. Any signal peptide may be used, including a signal peptide that would naturally direct secretion of an antibody, or other signal peptides derived from animal, plant or microbial secreted or ER-localised proteins. Many such sequences are well known to those skilled in the art. Particularly preferred is the PRIa leader sequence (Firek et al; Plant Mol. Biol, 23 861-870 (1993)).

Where it is desired to direct an antibody to the endoplasmic reticulum, and to retain it there, a signal sequence is provided, as above, and additionally an ER-retention signal is provided. Conveniently, this takes the form of a C-terminal polypeptide extension comprising the sequence Lys-Asp-Glu-Leu (KDEL). Alternatively, the KDEL sequence may be introduced by mutation of the residues at the C-terminal end of the antibody, or fragment of derivative thereof.

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cell, one or more DNA sequences encoding a heavy chain immunoglobulin or an active fragment or derivative thereof or one or more sequences encoding a protein functionally equivalent thereto and a transcriptional and translational termination regulatory region functional in a plant cell.

The promoter and termination regulatory regions will be functional in the host plant cell and may be heterologous (that is, not naturally occurring in the host plant) or homologous (derived from the plant host species) to the plant cell and the DNA sequence. Suitable promoters which may be used are described above.

The termination regulatory region may be derived from the 3' region of the gene from which the promoter was obtained or from another gene. Suitable termination regions which may be used are well known in the art and include *Agrobacterium tumefaciens* nopaline synthase terminator (*Tnos*), *Agrobacterium tumefaciens* mannopine synthase terminator (*Tmas*) and the *CaMV* 35S terminator (*T35S*). Particularly preferred termination regions for use according to the invention include the tobacco ribulose biphosphate carboxylase small subunit termination region (*TrbcS*) or the *Tnos* termination region.

Such DNA constructs may suitably be screened for activity by transformation into a host plant via *Agrobacterium* and screening for immunoglobulin levels.

In order to be able to select for plant cells that have integrated the construct, the expression construct may conveniently be joined to a marker which permits screening or selection, according to methods well known in the art.

Conveniently, the expression cassette according to the invention may be prepared by cloning the individual promoter/gene/ terminator units into a suitable cloning vector. Suitable cloning vectors are well known in the art, including such vectors as pUC (Norrand et al, (1983, Gene 26, 101-106), pEMBL (Dente et al (1983), Nucleic Acids Research, 11, 1645-1699), pBLUESCRIPT (available from Stratagene), pGEM (available from Promega) and pBR322 (Bolivar et al, (1977), Gene, 2, 95-113). Particularly useful cloning vectors are those based on the pUC series. The cloning vector allows the DNA to be amplified or manipulated, for example by joining sequences. The cloning sites are preferably in the form of a polylinker, that is a sequence containing multiple adjacent restriction sites, so as to allow flexibility in cloning.

Suitably, the nucleotide sequences for the genes may be extracted from the Genbank nucleotide database and searched for restriction enzymes that do not cut. These restriction sites may be added to the genes by conventional methods such as incorporating these sites in PCR primers or by sub-cloning.

Preferably the DNA construct according to the invention is comprised within a vector, most suitably an expression vector adapted for expression in an appropriate host (plant) cell. It will be appreciated that any vector which is capable of producing a recombinant plant comprising the introduced DNA sequence will be sufficient.

Suitable vectors are well known to those skilled in the art and are described in general technical references such as Pouwels et al, Cloning Vectors. A laboratory manual, Elsevier, Amsterdam (1986). Particularly suitable vectors include the Ti plasmid vectors.

Transformation techniques for introducing the DNA constructs according to the invention into host cells are well known in the art and include such methods as micro-injection, using polyethylene glycol, electroporation, or high velocity  
5 ballistic penetration. A preferred method for use according to the present invention relies on agrobacterium - mediated transformation.

After transformation of the plant cells or plant, those plant  
10 cells or plants into which the desired DNA has been incorporated may be selected by such methods as antibiotic resistance, herbicide resistance, tolerance to amino-acid analogues or using phenotypic markers.

15 Various assays, well known in the art, may be used to determine whether the plant cell shows the presence of the desired antibody, or fragment thereof. These include binding assay such as an ELISA or radio-immunoassay.

20 Plant cells transformed according to the invention may be grown in an appropriate culture or cultivated in soil or some other such suitable medium by methods well known in the art. It will be appreciated that the particular procedures adopted will vary depending on the plant species used, such variations  
25 being well within the knowledge of the average skilled practitioner.

These plant cells or plants may then conveniently be harvested and the desired antibody product processed using conventional  
30 extraction and purification techniques.

Seeds produced from modified plants containing the sequence coding for the desired antibody product can subsequently be grown to generate a progeny population of modified plants from  
35 which the desired product can be isolated.

Under some circumstances, it may be desirable to retain the antibody product with the plant rather than extracting and isolating the product. Where the modified plant or part thereof concerned is edible, for example, an antibody which has an effect on ingestion can conveniently be introduced in the form of a food product. In particular, edible plants or parts thereof, modified to incorporate an antibody or fragment or derivative thereof capable of binding a pre-selected antigen may conveniently be used in a method of passively immunising an animal, preferably a mammal and in particular a human, against said antigen. Particularly suitable antigens in this case will be pathogenic organisms or derivatives or parts thereof.

In addition to well-established application in the areas of diagnosis, therapy and purification, antibodies and fragments or derivatives thereof according to the present invention may suitably be used in methods to modify the properties of the plant in which they are produced. For example, the expression in a plant of functional anti-viral antibody can be useful in reducing pathogenicity and so provide a method of producing viral resistance. Alternatively, the introduction of antibodies into plants may be used to alter or interfere with plant metabolism by modulating the function of proteins present in the plant such as plant antigens and/or block phytohormones and metabolites.

The Invention will be further illustrated by means of the following examples which are provided by way of illustration only.

Techniques used for the modification and analysis of DNA materials were performed using standard procedures well known in the art, as described, for example, in Sambrook et al,

"Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor, Laboratory Press 1989 (hereinafter Sambrook), unless otherwise indicated.

- 5    Restriction sites are underlined.    HCV denotes heavy chain variable domain.

### EXAMPLES

10    Example 1: Construction of HCV expression vectors

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15    The construction of the HCV plant expression plasmids involved several cloning steps. The anti-RR6 HCV gene sequence was isolated from male llamas immunised with BSA-RR6 (as described in WO 99/23221). The sequence of the DNA fragment used in the following example (HCV33: SEQ. ID. No. 1) is given in Figure 1.

#### pSJ.30

20    The plasmid pSJ.30 is a derivative of the binary vector pGPTV-KAN (Becker et. al., Plant Mol. Biol. 20: 1195-1197, 1992) modified as follows: An approximately 750 bp (Sac I, T4 DNA polymerase blunted - Sal I) fragment of pJIT60  
25    (Guerineau et al., Plant Mol. Biol. 18: 815-818, 1992) containing the duplicated cauliflower mosaic virus (CaMV) 35S promoter (denoted 2x35S; Cabb-JI strain, equivalent to nucleotides 7040 to 7376 duplicated upstream of 7040 to 7433, Frank et. al., Cell 21: 285-294, 1980) was cloned  
30    into the Hind III (klenow polymerase repaired) - Sal I sites of pGPTV-KAN to create pSJ.30.

#### pPV.3



The shuttle vector pPV.3 was constructed by removing the HindIII/EcoRI multiple cloning site from pUC19 and replacing it by a synthetic DNA fragment, destroying the original EcoRI and HindIII sites and introducing a SgfI, HindIII, KpnI, EcoRI and XbaI restriction site. The new insert was constructed by annealing the synthetic oligonucleotides PCR.624 (SEQ. ID. No. 12) and PCR.625 (SEQ. ID. No. 13) (Table 1) yielding the insert sequence:

10 PCR.624

SgfI    HindIII    KpnI    EcoRI    XbaI  
 5' -AGCTGCGATCGCAAGCTTGGTACCGGAATTCTCTAGA-3'  
 3' -CGCTAGCGTTTCGAACCATGGCCCTTAAGAGATCTTTAA-5'

PCR.625

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#### pPV.5

The KpnI/EcoRI insert from pSJ.30 containing the 2x35S-promoter sequence upstream of the GUS gene, followed by the Nos terminator sequence was inserted into the KpnI/EcoRI opened pPV.3 vector yielding the intermediate vector pPV.5.

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#### pPV.5L

25 The GUS coding sequence was removed from the intermediate vector pPV.5 as a SalI/SacI fragment and replaced by a synthetic DNA fragment introducing a NcoI, NheI and MuiI restriction site, while leaving the original SalI/SacI sites intact. The new insert was constructed by annealing the synthetic oligonucleotides PCR.626 (Seq. ID. No. 14) and PCR.627 (Seq. ID. No. 15) (Table 1) yielding the insert sequence:

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SalI   NcoI                    NheI       MunI       SacI  
5' - TCGACCCATGGCCCGCTAGCCAATTGGAGCT - 3'  
3' - GGGTACCGGGCGATCGGTAAACC - 5'

pPV.5LN

10 The sequence present immediately 5' of the start codon ATG in  
this vector (CCACCATGG) was replaced by the plant Kozak  
sequence TAAACCATGG using PCR. Oligonucleotides PCR.640 (Seq.  
ID. No. 16) and PCR.641 (Seq. ID. No. 17) (Table 1) was used  
to amplify the 189 bp 3' fragment of the 2x35S promoter from  
15 vector pCP031 (Engelen et. al., Plant Mol. Biol., 26, 1701-  
1710, 1994), modifying the Kozak sequence via PCR.641. pCP031  
and the amplified fragment (SEQ. ID. No. 18) were digested  
with HindIII/EcoRV and EcoRV/NcoI respectively and used to  
replace the promoter present in the intermediate vector  
20 pPV.5L. After verification of the sequence by sequencing with  
primer Seq. ID. No. 19 the vector pPV.5LN was used in  
subsequent cloning steps.

PCR.640/PCR.641 amplification product (SEQ. ID. No. 18)

PCR. 640

CCACCCACGAGGGAACATCGTGGAAAAAGAAGACGTTCCAACCACGTCTTCAAAGCAAGT  
GGTGGGTGCTCCCTTGTAGCACCTTTTTCTTCTGCAAGGTTGGTGCAGAAGTTTCGTTC

30 EcoRV  
GGATTGATGTGATATCTCCACTGACGTAAGGGATGACGCACAATCCCACTATCCTTCGCA  
CCTAACTACACTATAGAGGTGACTGCATTCCTACTGCTTGTTAGGGTGATAGGAAGCGT

NcoI

35 AGACCCCTTCCTTTATATAAGGAAGTTCATTTCATTTGGAGAGGACCTCGAGTGTAAACCA

TCTGGGAAGGAAATATATTCTTCAAGTAA**AGTAAACCTCTCCTGGAGCTCACATTTGGT**

PCR.641

5 TGGGAATTC

**ACCCTTAAG**

pPV.5LN-PRIa-HCV33-myc

10 The PstI/EcoRI HCV33-myc fragment from pPIC.HCV33-myc  
(described in WO 99/23221 was inserted into the NcoI/MunI  
opened vector pPV.5LN together with a 105 bp NcoI/PstI  
fragment encoding the PRIa leader sequence allowing the in-  
frame fusion with the 5' framework-1 sequences of the HCV  
15 fragment.

The NcoI/PstI PRIa leader (Firek *et. al.*, Plant Mol. Biol.,  
23, 861-870, 1993) sequence (including the first 4 amino  
acids of the HCV fragment (QVQL)) was constructed by annealing  
20 the synthetic oligonucleotides PCR.293 (Seq. ID. No. 20),  
PCR.294 (Seq. ID. No. 21), PCR.295 (Seq. ID. No. 22), PCR.296  
(Seq. ID. No. 23), and PCR.297 (Seq. ID. No. 24) (Table 1).  
The product of the annealing reaction was cloned as a  
HindIII/PstI fragment into pUC.19. After verification of the  
25 sequence of the synthetic gene fragment by sequencing with  
primers Seq. ID. No. 25, 26 the NcoI/PstI fragment was excised  
and used in subsequent cloning reactions.

NcoI/PstI fragment encoding PRIA leader sequence  
30 (DNA Sequence Seq. ID. No. 27, Protein Sequence Seq. ID. No.  
28)

008161 924260



NcoI

CCATGGCAAGCATCACAGCTTCACACCACTTTGTGTCAAGAAGCCAACTTCACTAGACACCA  
GGTACCGTTTCGTAGTGTGGAAGTGTGGTGAAACACAGTTCTTCGGTTTGAAGTGATCTGTGGT

M A S I T A S H H F V S R S Q T S L D T

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AATCAACCTTGTACAGATAGGACTCAGGAACCATACTCTGACTCACAATGGTTTAAGGGCT  
 TTAGTTGGAACAGTGTCTATCCTGAGTCCTTGGTATGAGACTGAGTGTACCAAATTCCCGA  
 K S T L S Q I G L R N H T L T H N G L R A

10 GTTAACAACTTGATGGGCTCCAATCAAGAACTAATACTAAGGTAACACCCAAGATGGCATC  
 CAATTGTTTGAAGTACCCGAGGTAGTTCTTGATTATGATTCCATTGTGGGTCTACCGTAG  
 V N K L D G L Q S R T N T K V T P K M A S

PstI

15 CAGAACTGAGACCAAGAGACCTGGATGCTCAGCTACCATTGTTTGTGGAAAACAGGTGCAGCTGCAG  
 GTCTTGACTCTGGTTCTCTGGACCTACGAGTCGATGGTAACAAACACCTTTGTCCACGTCGACGTC  
 R T E T K R P G C S A T I V C G K Q V Q L Q

pPV.5LN-HCV33-myc

20 The PstI/EcoRI HCV33-myc fragment from pPIC.HCV33-myc was  
 inserted into the NcoI/MunI opened vector pPV.5LN together  
 with a synthetic NcoI/PstI DNA fragment allowing the in-frame  
 fusion with the ATG start codon. The synthetic linker was  
 constructed by annealing the synthetic oligonucleotides  
 25 PCR.652 (Seq. ID. No. 36) and PCR.653 (Seq. ID. No. 37) (Table  
 1) yielding the insert sequence:

PCR.652

30 NcoI PstI  
 5' -CATGCAGGTGCAGCTGCA-3'  
 3' -GTCCACGTCG-5'

PCR.653

pPV.5LN-HCV33-myc-SEKDEL

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The HindIII/BstEII Promoter-leader-HCV33 fragment inserts from

pPV5LN-PR1a-HCV33-myc, pPV5LN-GBSS-HCV33-myc and pPV5LN-no leader-HCV33-myc was cloned into HindIII/MunI opened pPV5LN-PR1a-HCV33-myc vector together with a myc-SEKDEL encoding BstEII/EcoRI PCR fragment thus introducing the sequence SEKDEL  
 5 at the C-terminal end of the myc-tag, thus yielding pPV.5LN-PR1a-HCV33-myc-SEKDEL, pPV.5LN-GBSS-HCV33-myc-SEKDEL and pPV.5LN-No leader-HCV33-myc-SEKDEL. The PCR fragment was generated using the synthetic oligonucleotides PCR.300 (Seq. ID. No. 38) and PCR.690 (Seq. ID. No. 39) (Table 1) using  
 10 pPV.5LN-PR1a-HCV33-myc as a template.

#### pSJ.34

The BamHI restriction site of the pGPTV-KAN expression  
 15 vector containing the kanamycin resistance selectable marker gene (Becker et. al., Plant Mol. Biol., 20, 1195-1197, 1992) was destroyed by restricting with BamHI followed by a Klenow reaction.

#### 20 pPV.8 plant transformation vectors

The HindIII/EcoRI inserts from vectors pPV.5LN-PR1a-HCV33-myc, pPV.5LN-GBSS-HCV33-myc, pPV.5LN-No leader-HCV33-myc, pPV.5LN-PR1a-HCV33-myc-SEKDEL, pPV.5LN-GBSS-HCV33-myc-  
 25 SEKDEL and pPV.5LN-No leader-HCV33-myc-SEKDEL were inserted into the HindIII/EcoRI vector fragment of pSJ.34, yielding pPV.8-GBSS-HCV33-myc, pPV.8-No leader-HCV33-myc, pPV.8-PR1a-HCV33-myc-SEKDEL, pPV.8-GBSS-HCV33-myc-SEKDEL and pPV.8-No leader-HCV33-myc-SEKDEL respectively.

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#### pPV.5LN-2x35S-GBSS-scFv3299-hydrophilIII

The PstI/EcoRI (partial) fragment containing scFv3299-hydrophilIII (also known as scFv.KC in patent filing WO  
 35 96/27612 and described in more detail in WO 99/27386) was

inserted into the NcoI/MunI opened vector pPV.5LN together with a 252 bp NcoI/PstI fragment encoding the GBSS leader sequence (as described for pPV.5LN-GBSS-HCV33-myc) allowing the in-frame fusion with the 5' framework-1 sequences of the HCV fragment. By way of explanation, the sequence of scFv3299 is disclosed in WO96/27612 (wherein it is described as FvKC-II), but with the BstEII-SacI linker fragment replaced with a BstEII-SacI fragment encoding a flexible linker yielding the sequence:

10 TVTVSSGGGGSGGGSGGGSDIELT (Seq. ID. No. 40). The DNA sequence of the linker is described by Jackson *et. al.* (Selection of variants of antibodies and other protein molecules using phage display on the surface of bacteriophage fd. : in Protein engineering: A Practical Approach, eds. Rees, Sternberg and Wetzel, publ. IRL Press, Oxford, 1992)

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pSJ.34-2x35S-GBSS-scFv3299-hydrophilII

20 The HindIII/EcoRI 2x35S promoter-GBSS-scFv3299-hydrophilII fragment of pPV.5LN-2x35S-GBSS-scFv3299-hydrophilII was inserted into the HindIII/EcoRI vector fragment of pSJ.34.

pSJ.89

25 A HindIII/SacI fragment containing (1x) 35S promoter-GUS.int (Vancanneyt *et. al.*, Mol. Gen. Genet, 220, 245-250, 1990) was inserted into the HindIII/SacI vector fragment of pSJ.34. This plant transformation vector with

30 Kan resistance contains the 35S promoter driving the GUS.int gene.

pSJ.103

The CERV promoter was isolated by PCR from infected *Dianthus barbatus* leaf material obtained from Dr Rene van der Lugt, Research Institute for Plant Protection (IPO-DLO), Binnehaven 5, P.O. Box 9060, NL-6700 GW, Wageningen, Netherlands, using the primers:

The approximately 380 bp fragment (corresponding to nucleotides 6737-7118 of Hull et. al., EMBO Journal, 5, 3083-3090, 1986) was amplified with oligonucleotides CERV1 (Seq. ID. No. 41) and CERV2 (14 nucleotides downstream of the transcription start site) (Seq. ID. No. 42) (Table 1) and was cloned into the TA cloning vector pT7Blue (Invitrogen) with the promoter in the same orientation as the T7 promoter. Sequence analysis showed that the isolated CERV promoter differed from the published sequence at several positions (T-C at 6790, C-T at 6826, A-G at 6872 and T-A at 6729) the most significant of which was a small deletion in a polyA tract in the 5' untranslated leader (4A's vs. 9A's in the published sequence). These changes probably represent differences in virus isolates rather than PCR errors but these cannot be ruled out.

A HindIII - BamHI fragment from pSJ.103 was used as the source of the promoter in plant transformation vectors. Since the HindIII site was 13 nucleotides downstream of the CERV1 oligonucleotide, this sequence was not present in the promoter.

### 30 pSJ.108

The HindIII/BamHI CERV promoter fragment of pSJ.103 was inserted into the HindIII/BamHI vector fragment of pSJ.89. This plant transformation vector with Kan resistance contains the CERV promoter driving the GUS.int gene.



pPV.7

The HindIII/EcoRI insert from pSJ.108 containing the CERV  
 5 promoter sequence upstream of the GUS gene, followed by  
 the Nos terminator sequence was inserted into the  
 HindIII/EcoRI opened pPV.3 vector yielding the  
 intermediate vector pPV.7.

10 pPV.7L

The GUS coding sequence was removed from the intermediate  
 vector pPV.7 as a BamHI/SacI fragment and replaced by a  
 synthetic DNA fragment introducing a NcoI, NheI and MunI  
 15 restriction site, while leaving the original BamHI/SacI  
 sites intact. The new insert was constructed by annealing  
 the synthetic oligonucleotides PCR.628 (Seq. ID. No. 43)  
 and PCR.629 (Seq. ID. No. 44) (Table 1) yielding the  
 insert sequence:

20

PCR.628

BamHI NcoI NheI MunI SacI  
 5' -GATCCCATGGCCCGCTAGCCAATTGGAGCT-3'  
 3' -GGTACCGGGCGATCGGTTAACC-5'

25

PCR.629

pPV.7LN

The sequence present immediately 5' of the start codon ATG  
 30 in this vector (GATCCCATGG) was replaced by the plant  
 Kozak sequence TAAACCATGG by replacing the BamHI/NheI  
 fragment present in pPV.7L with a synthetic fragment  
 constructed by annealing the synthetic oligonucleotides  
 PCR.645 (Seq. ID. No. 45) and PCR.646 (Seq. ID. No. 46)  
 35 (Table 1) yielding the vector pPV.7LN.

PCR.645

BamHI                      NcoI                      NheI  
 5' -GATCCACCTCGAGTGTAAACCATGGCCCG-3'  
 5                      3' -GTGGAGCTCACATTTGGTACCGGGCGATC-5'

PCR.646

pPV.7LN-GBSS-HCV33-myc

10 The PstI/MunI HCV33-myc fragment of pPIC.HCV33-myc was  
 inserted into the NcoI/MunI opened vector pPV.7LN together  
 with a 252 bp NcoI/PstI fragment encoding the GBSS leader  
 sequence (as described for pPV.5LN-GBSS-HCV33-myc)  
 allowing the in-frame fusion with the 5' framework-1  
 15 sequences of the HCV fragment.

pPV.7LN-GBSS-Clone1-His6-myc

20 The Clone1-His6-myc sequence was amplified by PCR using  
 oligonucleotides PCR.288 (Seq. ID. No. 47) and JW HCV2  
 (Seq. ID. No. 48) (Table 1) from pHEN.Clone1-His6-myc.  
 The PstI (partial)/MunI fragment was inserted into the  
 NcoI/MunI opened vector pPV.7LN together with a 252 bp  
 NcoI/PstI fragment encoding the GBSS leader sequence (as  
 25 described for pPV.5LN-GBSS-HCV33-myc) allowing the in-  
 frame fusion with the 5' framework-1 sequences of the HCV  
 fragment. The DNA sequence of clone 1 (Seq. ID. No 5,  
 Figure 6) was then verified with sequencing primers, Seq.  
 ID. No. 19,49 (Table 1).

30

pPV.7LN-GBSS-Clone46-His6-myc

The Clone46-His6-myc sequence was amplified by PCR using  
 oligonucleotides PCR.288 and JW HCV2 from pHEN.Clone46-  
 35 His6-myc. The PstI/MunI fragment was inserted into the

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NcoI/MunI opened vector pPV.7LN together with a 252 bp NcoI/PstI fragment encoding the GBSS leader sequence (as described for pPV.5LN-GBSS-HCV33-myc) allowing the in-frame fusion with the 5' framework-1 sequences of the HCV fragment. The DNA sequence of clone 46 (Seq. ID. No. 6, Figure 12) was then verified by sequencing with primers, Seq. ID. No. 19,49 (Table 1).

pPV.7LN-Clonel8-His6-myc

10

The Clonel8-His6-myc sequence was amplified by PCR using oligonucleotides PCR.288 and JW HCV2 from pHEN.Clonel8-His6-myc. The NcoI/MunI fragment was inserted into the NcoI/MunI opened vector pPV.7LN allowing the in-frame fusion with the 5' framework-1 sequences of the HCV fragment. The DNA sequence of clone 18 (Seq. ID. No. 8, Figure 14) was then verified by sequencing with primers, Seq. ID. No. 19,49 (Table 1).

20 pPV.7LN-Clonel8-His6-myc-SEKDEL

The Clonel8-His6-myc sequence was amplified by PCR using oligonucleotides PCR.288 and JW HCV1 (Seq. ID. No. 50) (Table 1; to generate 3' SEKDEL endoplasmic retention signal) from pHEN.Clonel8-His6-myc. The NcoI/MunI fragment was inserted into the NcoI/MunI opened vector pPV.7LN allowing the in-frame fusion with the 5' framework-1 sequences of the HCV fragment. The DNA sequence of Clonel8-His6-myc-SEKDEL (Seq. ID. No. 10, Figure 15) was then verified by sequencing with primers, Seq. ID. No. 19,49 (Table 1).

pSJ.35

The BamHI restriction site of the pGPTV-HYG expression vector containing the hygromycin resistance selectable marker gene (Becker et. al., Plant Mol. Biol., 20, 1195-1197, 1992) was destroyed by restricting with BamHI  
5 followed by a Klenow reaction.

pSJ.35-CERV plant transformation vectors

10 The HindIII/EcoRI inserts from vectors pPV.7LN-GBSS-HCV33-myc, pPV.7LN-GBSS-Clone1-His6-myc, pPV.7LN-GBSS-Clone46-His6-myc, pPV.7LN-Clone18-His6-myc and pPV.7LN-Clone18-His6-myc-SEKDEL were inserted into HindIII/EcoRI vector fragment of pSJ.35, yielding pSJ.35-CERV-GBSS-HCV33-myc,  
15 pSJ.35-CERV-GBSS-Clone1-His6-myc, pSJ.35-CERV-GBSS-Clone46-His6-myc, pSJ.35-CERV-Clone18-His6-myc and pSJ.35-CERV-Clone18-His6-myc-SEKDEL respectively.

pCJ.102

20 Oligonucleotides 167 (Seq. ID. No. 51) and 168 (Seq. ID. No. 52) (Table 1) were used to amplify the 460bp 5' fragment of the GUS gene (from vector pSJ.34) and modifying the 5' end to create a NcoI site and thus  
25 modifying the second amino acid.

The PCR amplified fragment was digested with NcoI and EcoRV and inserted into HindIII/EcoRI vector fragment of pSJ.34 together with the HindIII/NcoI 2x35S promoter fragment of pPV.5LN and the EcoRV/EcoRI 3'GUS-Nos  
30 terminator fragment of pSJ.34, yielding the vector pCJ.102. The DNA sequence of the amplified fragment was verified by sequencing with primer Seq. ID. No. 53 (Table 1).

35 pSJ.35-Lat52-Clone18-His6-myc

The NcoI/EcoRI Clonel8-His6-myc-Nos fragment of pPV.7LN-Clonel8-His6-myc was inserted into the SalI/EcoRI vector fragment of pSJ.35 together with the SalI/NcoI Lat52 promoter fragment of pLAT52-7 (Twell et. al., Development, 109, 705-13, 1990).

pSJ.35-Lat52-Clonel8-His6-myc-SEKDEL

10 The NcoI/EcoRI Clonel8-His6-myc-SEKDEL-Nos fragment of pPV.7LN-Clonel8-His6-myc-SEKDEL was inserted into the SalI/EcoRI vector fragment of pSJ.35 together with the SalI/NcoI Lat52 promoter fragment of pLAT52-7 (Twell et. al., Development, 109, 705-13, 1990).

pSJ.35-Lat52-HCV33-myc-SEKDEL

15 The PstI/SacI HCV33-myc-SEKDEL fragment of pPV.8-GBSS-HCV33-myc-SEKDEL was inserted into the SalI/SacI vector fragment of pSJ.35 with the SalI/PstI Lat52 promoter-5' antibody gene fragment of pSJ.35-Lat52-Clonel8-His6-myc.

Example 2: Transformation and cultivation of *Nicotiana Tabacum*

25 Cells of the agrobacterium strain LBA4404 were transformed with the described plasmids using the freeze thaw method (Plant Molecular Biology Manual PMAN A3/7).

30 Seeds of *Nicotiana Tabacum* (var. Petit Havana SR1) were surface sterilised for 10 minutes in a solution of 10% sodium hypochlorate, rinsed 3 times in sterile distilled water and planted in Murashige & Skoog (MS) basal medium + 3% sucrose + 0.9% agar.

After 2 weeks growth seedlings were thinned to 2 per vessel; sterile plantlets were maintained by monthly shoot cuttings onto MS basal media +3% sucrose + 0.9% agar.

- 5 Discs were punched from leaves of these plants using a sterile cork borer, then incubated for 10 minutes in a culture of the agrobacterium strain LBA4404 containing the described plasmids which had been grown overnight in Lennox Broth (5g/l NaCl, Yeast Extract 10 g/l, 10 g/l Bacto Tryptone, 15g/l agar). The  
10 overnight culture was spun down for 10 minutes at 3000G and the growth media replaced by re-suspending the cells in MS Basal medium +3% sucrose.

- Discs were removed from the culture and blotted dry on sterile  
15 filter paper before plating face down on a nurse culture plate of tobacco cells. This plate was prepared by adding 2ml of a cell suspension culture of Nicotiana Bethiana onto a petri dish containing 25ml of MS Salts, B5 vitamins (1mg/l Nicotinic acid, 1mg/l Pyridoxine, 10mg/l Thiamine, 100mg/l Inositol)  
20 +1mg/l 2-4 D, 0.2 mg/l BAP & 0.8% agar. The cells were swirled to cover the agar then covered with a sterile filter paper disc.

- Infected leaf discs were incubated at 26°C in a light  
25 intensity of 2000 lux for 2 days, then removed from the nurse culture plates to selection media (MS Basal media +3% sucrose, 0.2mg/l IAA, 1mg/l BAP, 0.9% agar + cefotaxime 500mg/l and kanamycin 100 mg/l or hygromycin B 20mg/l). Plates were then incubated at 3000 lux, 26 C, 16 hours day/8 hour night and  
30 transferred every 2 weeks to fresh media (as previously). Shoots that appeared from the cut edges of the discs were removed to MS Basal media + 3% sucrose, cefotaxime 500mg/l and kanamycin 100mg/l or hygromycin B 20mg/l to root. Leaf samples were taken from rooted shoots for testing as described  
35 in Example 3, 4, 5, 7, 8 & 9.

Example 3: Extraction and detection of HCV materialExtraction of leaf tissue for analysis of anti-RR6 antibody  
5 (denoted HCV 33)

Leaf tissue was homogenised with Polytron probe in 2ml microcentrifuge tubes in the presence of 4ml extraction buffer (200mM Tris-HCl pH7.5, 5mM EDTA, 0.1% Tween 80, 1 mM PMSF {PhenylMethylSulfonylFluoride}) per 1gr leaf material.

10 Solid material was removed from the homogenate by centrifugation (10 min 13,000g). The cleared supernatant was stored at -20°C. Total protein concentration was determined using the PIERCE BCA Protein Assay Reagent detection system.

15 Extraction of tomato fruit tissue for analysis of anti-RR6 antibody (denoted HCV33)

Fruit were dissected into peel, flesh and columella (centre of fruit), frozen and ground with a mortar and pestle in liquid nitrogen. Extracts were prepared as for  
20 leaf tissue above.

Extraction of leaf tissue for analysis of anti-potato  
SBEII antibodies (denoted Clone1 and Clone46)

25

Leaf tissue was homogenised with Polytron probe in 2ml microcentrifuge tubes in the presence of 4 ml SBE extraction buffer (100mM Tris-HCL pH7.5, 5mM EDTA, 2.5mM 1,4-dithiothreitol, 0.1% (w/v) sodium metabisulphite, 5%  
30 (v/v) glycerol, 2.5% (w/v) polyvinylpolypyrrolidone, 1mM PMSF, 1 tablet Complete (Roche) per 50ml) per 1gr fresh weight. Solid material was removed from the homogenate by centrifugation (10 min 13,000g). The cleared supernatant was stored at -20°C. Total protein concentration was

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determined using the BioRad Protein Assay Reagent detection system.

Extraction of leaf tissue for analysis of anti-GUS

5 antibody (denoted Clone18)

As described for analysis of anti-RR6 antibody, except GUS extraction buffer (50mM phosphate pH 7.0, 10mM EDTA, 0.1% Triton X-100, 1 mM DTT).

10

Detection of RR6 binding activity

96 well ELISA plates (Greiner) were coated overnight at 4°C with 100µl per well of BSA-RR6 conjugate (10µg/ml) in 15 0.05 M NaCO<sub>3</sub> buffer pH9.5. Following three washes with tap water, the plates were incubated for 1 hour at 37°C with 250µl per well of a blocking solution of 3% Marvel in TBST (20mM Tris-HCL pH7.5, 140mM NaCl, 0.1% Tween 20). Test plant extracts and appropriate standards (purified, 20 *P.pastoris* produced HCV33-myc standard) in duplicate were added to wells in a total volume of 100µl TBST, and the plates incubated at 4°C overnight. Unbound antibody fragment was removed by 6 washes with tap water. 100µl of the primary monoclonal anti-myc antibody No. 4111 (1µg/mL) in 25 1% Marvel/TBST were added to each well. Following incubation at 37°C for 1 hour, unbound antibody was removed by 6 washes with tap water and 100 µL of the secondary anti-mouse antibody conjugated alkaline phosphatase (1µg/mL) was added to each well. Following incubation at 30 37°C for 1 hour, unbound antibody was removed by 6 washes with tap water, and alkaline phosphatase activity was detected by adding 100µl of 1mg/ml pNPP in 1M diethanolamine/1mM MgCl<sub>2</sub>. Plates were incubated at room temperature for up to 5 hours before reading at 410nm on a 35 Dynex, MCX plate reader.

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Detection of SBEII binding activity

As for RR6 binding activity, except:

5

100µl/well of 5µg/ml recombinant potato SBEII was bound to the plate (Jobling *et al.*, Plant Journal, 18, 163-171, 1999) in PBS overnight at 4°C

10 Purified and quantified clone1-myc from E.coli was used as the standard.

Blocking buffer: 1% BSA in TBST, all incubations at room temperature.

15

100µl/well of 1:2,000 dilution of rabbit anti-llama IgG, as the primary detection antibody.

20

100µl/well of 1µg/mL goat anti-rabbit antibody conjugated alkaline phosphatase, as the secondary detection antibody.

SDS-PAGE and Western blotting

Plant extracts were analysed on 10% bis-acrylamide gel using the Bio-Rad mini-Protean II system according to manufacturers instructions. 40µg samples and appropriate standards (purified *P.pastoris* produced HCV33-myc) were diluted in 50µl 2xSDS-PAGE loading buffer (20% Glycerol, 20mM Tris-HCL pH 8.0, 0.5% SDS, 0.005% bromophenol blue). The samples were mixed, boiled (5 minutes) and centrifuged briefly before loading onto the gel. The gels were run at 100V for 1 hour in 1x running buffer (25mM Tris, 200mM glycine, 1% SDS).

Proteins were transferred onto nylon membranes (Millipore) by semi dry blotting at 25V, 80mA for 45 minutes in semi-dry blotting buffer (50mM Tris, 40mM glycine, 0.04% SDS, 20% methanol). The membranes were then dried overnight at room temperature. The membranes were incubated in 3% Marvel, TBST for 10 minutes then in 1µg/mL mouse anti-myc antibody in 1% Marvel, TBST for 60 minutes at room temperature with constant agitation. Unbound antibody was removed by washing 6 times in TBST at room temperature with constant agitation. The blot was then incubated with the secondary antibody (rabbit anti-mouse IgG conjugated to alkaline phosphatase) in 1% Marvel, TBST for 60 minutes at room temperature with constant agitation. Unbound antibody was removed by washing 6 times in TBST at room temperature with constant agitation. Bound antibody was detected by incubating the filters in 10mL of 30mg/mL NBT and 15mg/mL BCIP. Washing the filters with water stopped the colour reaction.

## 20 EM studies

### Step 1: Sample preparation:

Leaf samples were fixed using 1% Paraformaldehyde+ 0.05% glutaraldehyde in 0.05M sodium phosphate buffer pH6.8, for 4hrs at 4°C, and then washed in phosphate buffer overnight at 4°C. Samples were embedded by first dehydrating the samples through alcohol, 50, 70, 90% (15mins) and finally absolute alcohol (2x30mins). They were then placed in hydrophilic resin [6 parts LRGold resin + 4 parts GMA (low acid) + 0.1% benzoin ethyl ether (UV activator). Resin was changed several times over period of three days. Finally, samples were embedded, using freshly prepared resin, in flat bottomed plastic moulds, allowing the resin to polymerise for 24hrs at room temperature, under nitrogen gas, using UV light (360nm).

## Step 2: Staining:

Ultrathin leaf tissue sections were collected on collodion (2% in amyl acetate) coated nickel e.m. grids. Grids were floated  
5 on 20 $\mu$ l aliquots of TBS/BSA [20mM Tris/HCL buffer pH7.6 +  
0.23M NaCl +1 bovine serum albumin] for 20mins at room  
temperature, following by incubation with 20 $\mu$ l aliquots of  
primary antibody (anti-myc or anti-hydrophil II, 1 $\mu$ g/ml) in  
TBS/BSA +0.05% Tween 20 for 18hrs at room temperature. Grids  
10 were washed thoroughly using TBS. The primary antibody was  
detected by incubating the grids with 20 $\mu$ l aliquots of  
secondary antibody/gold conjugate diluted in TBS/BSA (Goat a  
Mouse IgG; 5nm diluted 1:200, 10nm diluted 1:100 20nm diluted  
1:50) for 60mins at room temperature. The grids were washed  
15 thoroughly, using TBS followed by washes with deionised water  
and dried. Some grids were silver enhanced, prior to  
poststaining with 2% aqueous Uranyl Acetate, followed by Lead  
Citrate. Photographs were taken using either a Jeol 100CX  
Mk2 transmission electron microscope at 60,000 magnification  
20 (Figure 4) or a Jeol 1220 transmission electron microscope at  
40,000 magnification (Figure 11).

Assay of SBE activity

25 All protein samples were diluted to a concentration of  
1mg/mL using SBE extraction buffer. SBE was assayed using  
the phosphorylase a stimulation assay (Hawker *et al.*,  
Arch. Biochem. Biophys., 160, 530-551, 1974). The reaction  
was carried out at 30°C for 1h on 50 $\mu$ g protein in a final  
30 volume of 200 $\mu$ l and contained 100mM 2-(N-  
morpholino)ethanesulphonic acid (MES), 50mM [U-  
14C]glucose-1-phosphate (370MBq mole<sup>-1</sup>, ICN), 1.3U rabbit  
muscle phosphorylase a (Sigma), 50 $\mu$ l plant extract at  
1mg/ml protein. Glucose polymers were precipitated and

washed as described by Hawker et al., Arch. Biochem. Biophys., 160, 530-551 (1974). The final pellet was dissolved in DMSO and counted with ReadySafe-scintillation cocktail (Beckmann).

5

#### Assay of GUS activity

All protein samples were diluted to a concentration of 1 mg/ml using GUS extraction buffer. 10µl samples were then added to 190µl of 1 x reaction buffer (50 mM phosphate pH 7.0, 0.1% Triton X-100, 1 mM DTT, 1 mM p-nitrophenyl β-D-glucuronide) contained in the well of a flat-bottomed microtitre plate. The reaction was left to proceed at 37°C until the wells turned visibly yellow. At this point, 80µl of stop solution (2.5M 2-amino-2-methyl propanediol) was added and the plate read at 410nm using a Dynex plate reader. GUS activity was calculated based on positive control wells to which known quantities of GUS were added.

#### 20 Histochemical staining for GUS activity

Tobacco pollen was stained for GUS activity in microtitre wells for up to 1 hour in 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid according to the method of Jefferson et al., EMBO Journal 6: 3901-7, (1987). The buffer was modified by the use of histochemical staining buffer comprising 100 mM sodium phosphate pH 7.0, 10 mM EDTA, and 0.1% (v/v) Triton X-100 (Stomp, In: *Editorial comments*, Vol. 16, No.5. Cleveland: united State Biochemical, 1990). To minimise spread of the stain to surrounding pollen grains, 5mM potassium ferricyanide was added to localise staining (Lojda, Histochemie 22: 347-61, 1970; Mascarenhas and Hamilton, Plant Journal 2: 405-8, 1992).

#### 35 Example 4: Expression of HCV antigen binding activity in the

## cytoplasm

Small samples of the leaves of 10 individual Tobacco plants transformed with the expression vector pPV.8-HCV33-myc were tested for the presence of anti-RR6 binding activity using ELISA. One of the tested samples showed low levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity present in this clone (No. 943.37) using SDS-PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed as 0.001-0.002% of total protein present (Figure 2 and Table 2).

Example 5: Expression of HCV antigen binding activity in plastids

Small samples of the leaves of 15 individual Tobacco plants transformed with the expression vector pPV.8-GBSS-HCV33-myc were tested for the presence of anti-RR6 binding activity using ELISA. Seven of the tested samples showed high levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity present in these clones using SDS-PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed as 0.5-1.0% of total soluble protein present (Figure 3 and Table 2). From the Western blot it can be concluded that the construct is processed correctly and that the GBSS leader is removed from the HCV fragment. Localisation of the HCV33 material in the chloroplasts was confirmed via immunohistology studies (Figure 4).

The fruit of 10 individual tomato plants transformed with the expression vector pPV.8-GBSS-HCV33-myc were picked 15 days post-breaker (red stage), dissected and tested for the presence of anti-RR6 binding activity using ELISA. 5

of tested fruit expressed levels of RR6-binding activity which, by comparison to a standard curve of purified HCV33 binding to RR6, were over 0.1% (one was over 0.2%) of total soluble protein (Figure 5 and Table 2).

5

Similar high levels of expression were obtained with an HCV antibody of different specificity. Small samples of the leaves of 30 individual tobacco plants transformed with the expression vector pSJ.35-CERV-GBSS-Clone1-His6-myc were tested for the presence of anti-SBEII binding activity using ELISA. 3 of the tested plants expressed levels of SBEII-binding activity over 0.1% total soluble protein (Figure 7 and Table 2).

15 Example 6: Disruption of plastid by scFv expression

Tobacco plants were transformed with expression vector pSJ34-2x35S-GBSS-scFv3299-hydrophilIII. After initial detection of high expression of hCG binding activity, these levels were lost - expression was unstable (not shown). Furthermore, immuno-histology studies (Figure 8) suggested that scFv expression had an adverse affect on plastid structure and function, perhaps due to the aggregation of multiple scFv molecules at the plastid membrane.

Example 7: Expression of HCV antigen binding activity in the apoplastic space

Small samples of the leaves of 21 individual Tobacco plants transformed with the expression vector pPV.8-PR1a-HCV33-myc were tested for the presence of anti-RR6 binding activity using ELISA. 12 of the tested samples showed low levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity present using showed that HCV33-myc was

expressed as 0.0002-0.00074% of total protein present (Figure 9 and Table 2).

Example 8: Expression of HCV33 containing the ER retention  
5 signal SEKDEL

Small samples of the leaves of 22, 23 and 12 individual Tobacco plants transformed with the expression vectors pPV.8-  
no leader-HCV33-myc-SEKDEL, pPV.8-PR1a-HCV33-myc-SEKDEL,  
10 pPV.8-GBSS-HCV33-myc-SEKDEL respectively were tested for the presence of anti-RR6 binding activity using ELISA. None, 16 and 3 of the tested samples respectively, showed medium to high levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity present in these clones using SDS-  
15 PAGE/Western blot analysis, ELISA and total protein concentration measurement showed that HCV33-myc was expressed as 0, 0.4 and 0.05% of total protein present respectively (Figure 10 and Table 2). Localisation of the HCV33 material, which was directed to the secretory pathway by using the PR1a  
20 leader sequence, in the ER was confirmed using immunohistology studies (Figure 11).

Example 9: Effect of neutralising HCV expression on SBE  
25 activity in tobacco plants

Samples of the leaves of 30 individual tobacco plants transformed with expression vector pSJ.35-CERV-GBSS-Clone46-His6-myc were assayed for SBE activity. Compared to the effect of the HCV33 anti-RR6 antibody in 30 control  
30 plants transformed with expression vector pSJ.35-CERV-GBSS-HCV33-myc, the specific anti-potato SBEII antibody Clone46 significantly reduced the SBE activity in the tobacco plants (Figure 13), indicating that the antibody has neutralised the enzyme activity.

Example 10: Effect of neutralising HCV expression on GUS  
activity in tobacco leaves

5 A tobacco line already transformed with expression vector  
pCJ.102 and shown to contain GUS activity in leaf tissue  
was retransformed with the expression vector pSJ35-CERV-  
Clone18-His6-myc-SEKDEL. Samples from 27 individual  
transformed plants were assayed for GUS activity in  
10 comparison to control pCJ.102 tobacco lines that had not  
been retransformed. A graphical representation of the  
results from measuring GUS activity in control lines and  
in lines containing Clone 18 is shown in Figure 16. A  
difference between the two sets of data is clearly  
evident, indicating that the presence of the anti-GUS  
15 antibody has decreased GUS activity.

Example 11: Effect of neutralising HCV expression on GUS  
activity in tobacco pollen

20 The tobacco line *lat52-GUS*, containing the pollen specific  
promoter *lat52* driving *GUS* in a single copy homozygous  
state, was obtained as a gift from Dr. David Twell (Botany  
Department, University of Leicester, UK). This line was  
retransformed with expression vector pSJ.35-Lat52-Clone18-  
25 His6-myc-SEKDEL. Control lines were obtained by  
transforming *lat52-GUS* tobacco with pSJ.35-Lat52-HCV33-  
myc-SEKDEL. Pollen from 20 lines of each set of  
transformed tobacco were tested by histochemical staining  
for GUS activity. Scanned images showing the staining  
30 pattern obtained in a control line and a line containing  
anti-GUS VHH clone 18 are shown in Figure 17. It can quite  
clearly be seen that GUS activity has been 'knocked-out'  
by the presence of anti-GUS antibody. Half the pollen



retains GUS activity (shown by blue staining) due to segregation of the transgene at meiosis.

5 Example 12: Expression of whole heavy chain immunoglobulins in plants

Construction of HCV33-IgG2b expression vectors

10 pPV.5LN-HCV33-IgG2b

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A cDNA clone IgG2b (Vu et. al., Mol. Imm., 34, 16-17, 1997) was amplified by PCR using oligonucleotides 304 (Seq. ID. No. 58) and 305 (Seq. ID. No. 59), 306 (Seq. ID. No. 60) and 310 (Seq. ID. NO. 61) (Table 1). The amplified fragments were digested with BstEII/HincII and HincII/EcoRI respectively and inserted into NcoI/MunI opened vector pPV.5LN together with the NcoI/BstEII HCV33 fragment from vector pPV.5LN-HCV33-myc, yielding the vector pPV.5-HCV33-IgG2b. The DNA sequence of the amplified fragment was verified by sequencing with primers Seq. ID. No. 19, 53, 58, 60 and 62 (Table 1).

25 The predicted amino acid sequence contained two mutations at amino acid numbers 167 and 369 (Figure 18) as compared to the published sequence by Woolven et. al., Immunogen., 50, 98-101, 1999. Oligonucleotides 314 (Seq. ID. No. 63) and 307 (Seq. ID. No. 64) (Table 1) were designed to change the mutations at amino acids 167 (proline) and 369 (Stop codon) to a serine and lysine respectively.

30 The HCV33-IgG2b clone was then amplified by PCR using oligonucleotides 314 and 307 (Table 1). The amplified fragment was digested with SacI/EcoRI and inserted into HindIII/MunI opened vector pPV.5LN together with the HindIII/SacI fragment from vector pPV.5-HCV33-IgG2b, yielding the vector pPV.5LN-HCV33-IgG2b. The DNA sequence of the amplified fragment was verified

35

by sequencing with primers Seq. ID. No. No. 19, 53, 58, 60 and 62 (Table 1) (Figure 18).

pPV.5LN-PR1a-HCV33-IgG2b

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The BstEII/EcoRI HCV33-IgG2b fragment from vector pPV.5LN-HCV33-IgG2b was inserted into HindIII/EcoRI opened pPV.5LN together with the HindIII/BstEII promoter-PR1a-HCV33 fragment from vector pPV.5LN-PR1a-HCV33-myc.

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pPV.5LN-GBSS-HCV33-IgG2b

The BstEII/EcoRI HCV33-IgG2b fragment from vector pPV.5LN-HCV33-IgG2b was inserted into HindIII/EcoRI opened pPV.5LN together with the HindIII/BstEII promoter-GBSS-HCV33 fragment from vector pPV.5LN-GBSS-HCV33-myc.

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pPV.5LN-PR1a-HCV33-IgG2b-SEKDEL

Vector pPV.5LN-HCV33-IgG2b was amplified by PCR using oligonucleotides 306 and 308 (Seq. ID. No. 65) (Table 1; to generate 3' SEKDEL endoplasmic retention signal). The amplified fragment was digested with HincII/EcoRI and inserted into HindIII/EcoRI opened pPV.5LN together with the HindIII/HincII fragment from vector pPV.5LN-PR1a-HCV33-IgG2b. The DNA sequence of the amplified fragment was verified by sequencing with primers Seq. ID. No. 19, 63 (Table 1) (Figure 19).

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pPV.8 2x35S plant transformation vectors

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The HindIII/EcoRI inserts from vectors pPV.5LN-HCV33-IgG2b, pPV.5LN-PR1a-HCV33-IgG2b, pPV.5LN-GBSS-HCV33-IgG2b and pPV.5LN-PR1a-HCV33-IgG2b-SEKDEL were inserted into HindIII/EcoRI vector fragment of pSJ.34, yielding pPV.8-HCV33-IgG2b, pPV.8-PR1a-HCV33-IgG2b, pPV.8-GBSS-HCV33-IgG2b and pPV.8-PR1a-HCV33-IgG2b-SEKDEL respectively.

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As for RR6 binding activity, except:

100µl/well of 1:2,000 dilution of rabbit anti-llama IgG was used as the primary detection antibody.

Expression of HCV33-IgG2b antigen binding activity in the cytoplasm

Three plants chosen at random were selfed and allowed to set seed, the next generation was germinated and after 6 weeks small samples of leaf, stem and root were tested for the presence of anti-RR6 binding activity using ELISA. Two of the tested plants showed increased levels of RR6 binding activity as compared to the original analysis. Detailed analysis of the anti-RR6 binding activity showed that HCV33-IgG2b was expressed at its highest in stem tissues at a maximum level of 1.07% total protein present (Figure 21).

Expression of HCV33-IgG2b antigen binding activity in the plastids

Small samples of the leaves of 20 individual Tobacco plants transformed with the expression vector pPV.8-GBSS-HCV33-IgG2b were tested for the presence of anti-RR6 binding activity using ELISA. 10 of the tested samples showed low levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity showed that HCV33-IgG2b was expressed as 0.034-0.050% of total soluble protein present (Figure 22).

Three plants chosen at random were selfed and allowed to set seed, the next generation was germinated and after 6 weeks small samples of leaf, stem and root were tested for the presence of anti-RR6 binding activity using ELISA. All of the tested samples showed increased levels of RR6 binding activity as compared to the original analysis. Detailed analysis of the anti-RR6 binding activity showed that HCV33-IgG2b was expressed at its highest in leaf tissues at a maximum level of 9.26% total soluble protein present (Figure 23).

Expression of HCV33-IgG2b antigen binding activity in the apoplastic space

Small samples of the leaves of 20 individual Tobacco plants transformed with the expression vector pPV.8-PR1a-HCV33-IgG2b were tested for the presence of anti-RR6 binding activity using ELISA. 17 of the tested samples showed significant levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity showed that HCV33-IgG2b was expressed as 0.021-0.157% of total soluble protein present (Figure 24).

Three plants chosen at random were selfed and allowed to set seed, the next generation was germinated and after 6 weeks small samples of leaf, stem and root were tested for the presence of anti-RR6 binding activity using ELISA. All of the tested samples

showed increased levels of RR6 binding activity as compared to the original analysis. Detailed analysis of the anti-RR6 binding activity showed that HCV33-IgG2b was expressed at very high levels in all tissues tested, with the highest level found in leaf at a maximum of 13% total soluble protein present (Figure 25).

Expression of HCV33-IgG2b antigen binding activity in the endoplasmic reticulum

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Small samples of the leaves of 20 individual Tobacco plants transformed with the expression vector pPV.8-HCV33-IgG2b were tested for the presence of anti-RR6 binding activity using ELISA. 13 of the tested samples showed significant levels of RR6 binding activity. Detailed analysis of the anti-RR6 binding activity present using showed that HCV33-IgG2b was expressed as 0.02-0.165% of total soluble protein present (Figure 26).

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Three plants chosen at random were selfed and allowed to set seed, the next generation was germinated and after 6 weeks small samples of leaf, stem and root were tested for the presence of anti-RR6 binding activity using ELISA. All of the tested samples showed increased levels of RR6 binding activity as compared to the original analysis. Detailed analysis of the anti-RR6 binding activity showed that HCV33-IgG2b was expressed at very high levels in all tissues tested, with the highest level found in leaf at a maximum of 16.3% of total soluble protein present (Figure 27).

30 Example 13: Expression of anti-SBEII (clone68) antigen binding activity in tobacco directed to the plastids

Construction of CERV-GBSS-Clone68-myc expression vectors

35 pPV.7LN-GBSS-Clone68-His6-myc

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pSJ.35-CERV plant transformation vector

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### Detection of SBEII binding activity

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### Expression of anti-SBEII antigen binding activity in the plastids

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Table 1: DNA sequence of the synthetic oligonucleotides used in the examples.

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Code	SEQ. ID. No.	Nucleotide Sequence
PCR.624	12	AGCTGCGATCGCAAGCTTGGTACCGGGAATTCTCTAGA
PCR.625	13	AATTTCTAGAGAATTCCCGGTACCAAGCTTGCTTGCGATCGC
PCR.626	14	TCGACCCATGGCCCGCTAGCCAATTGGAGCT
PCR.627	15	CCAATTGGCTAGCGGGCCATGGG
PCR.640	16	CCACCCACGAGGGAACATCGTG
PCR.641	17	GAATTC CATGGTTTACACTCGAGGTCTCTCCAAATGA
172	19	CCGGCAACAGGATTCAATCTT
PCR.293	20	AGCTCCATGGGATTGTCTCTTTTCACAATTGCCTTCAT
PCR.294	21	TTCTTCTGTCTCTACACTTCTCTTATTCCTAGTA
PCR.295	22	ATATCCCCTCTTGCCGTGCCAGGTGCAGCTGCA
PCR.296	23	GCTGCACCTGGGCACGGCAAGAGTGGGATATTACTAGGAATAAGAGAA
PCR.297	24	GTGTAGAGACAAGAAGAAATGAAGGCAATTGTGAAAAGAGAACAAATCCC ATGG
152	25	GGAAACAGCTATGACCATGATTAC
191	26	TTTCCCAGTCACGACGTTGT
132	29	GACATCCCATGGCAAGCATCA
133	30	AAGCTTGTTAACAGCCCTTAA
134	31	AGGGCTGTTAACAACTTGAT
136	32	AGACTGCTGCAGCTGCACCTGCTTCCACAAACAATGGTAGCTG
197	33	AATTAACCCTCACTAAAGG
PCR.652	36	CATGCAGGTGCAGCTGCA
PCR.653	37	GCTGCACCTG
PCR.300	38	CGCAAGACCCCTCCTCTATATAAG
PCR.690	39	GAGCTCGAATTCCTATTATAGCTCATCTTTCTCTGAATTCAGATCCTCTT CTGAGATGAG
CERV1	41	ATCCTCAACTTCCAATCAGA
CERV2	42	TTCTTGAGAGATAGCTTGA
PCR.628	43	GATCCCATGGCCCGCTAGCCAATTGGAGCT
PCR.629	44	CCAATTGGCTAGCGGGCCATGG

PCR.645	45	GATCCACCTCGAGTGTAACCATGGCCCG
PCR.646	46	CTAGCGGGCCATGGTTTACACTCGAGGTG
PCR.288	47	ATTGCCTACGGCAGCCGCTG
JWHCV2	48	TCCAACCAATTGTTACTATGCGGCCCCATTAGATCCTCTTCTGAG ATGAG
164	49	GTCTGTCTAAAGTAAAGTAGATGCG
JWHCV1	50	TCCAACCAATTGTTATCATAGCTCATCTTTCTCACTATTAGATCC TCTTCTGAGATGAG
167	51	AGTCCCCCATGGTACGTCTGTAGAAACC
168	52	CGTTTTCGTCGTAATCACCATTCC
161	53	CGCAAGACCCTTCCTTTATATAAG
304	58	AGGGGACCCAGGTCACCGTCTCCTCAGAACC
305	59	GAGCTTTGTTGTTGACCTTGCAATTTGAACCTTTTCCC
306	60	CAAATGCAAGGTCAACAACAAAGCTC
310	61	TTCGAGCTCGAATTCTTATTATTTACCCGAAGACTGGGTGAT
303	62	CTGAGGAGACGGTGACCTGGGTCCCCT
314	63	AGCCCCCTGAGCTCCTGGGAGGGCCCTCAGTCTTCATCTTCCCCCG
307	64	TTCGAGCTCGAATTCTTATTATTTACCCGAAGACTGGGTGATGGATTCT GGGTGTAGTGG
308	65	TTCGAGCTCGAATTCTTATTATAGCTCATCTTTCTCAGATTTACCCGAAG ACTGGGTGATGGATTCTGGGTGTAGTGG

5 Table 2: Summary of HCV expression levels in tobacco (unless otherwise stated) detected. Maximum observed expression levels are expressed as percentage of active antibody in the total amount of soluble protein extracted.

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Construct	Compartment	Expression Level
pPV.8-no leader-HCV33-myc	Cytosol	0.002%
pPV.8-GBSS-HCV33-myc	Chloroplast	1.0%
pPV.8-PR1a-HCV33-myc	Apoplast	0.00074%
pPV.8-no leader-HCV33-myc-KDEL	Cytosol	<0.001%
pPV.8-GBSS-HCV33-myc-KDEL	Chloroplast	0.05%



pPV.8-PRIa-HCV33-myc-KDEL	ER	0.4%
pPV.8-GBSS-HCV33-myc	Plastid (Tomato fruit columella)	0.2%
pSJ.35-CERV-GBSS-Clon1-His6-myc	Chloroplast	0.2%

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